Investigating Interactions between Neurotactin, Amalgam and Lachesin in Insects, Three Proteins that Function in Neuronal Cell Adhesion

A Senior Honors Thesis

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by

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ABSTRACT

Neurotactin (Nrt) is a transmembrane protein that interacts with Amalgam (Ama), a secreted protein, to promote cell:cell adhesion during nervous system development in *Drosophila*. The Ama/Nrt cell adhesion process has been shown to be involved in axon-pathfinding. Lachesin (Lac), another cell surface protein, may also play a role in *Drosophila* neurite outgrowth. Lac and Ama proteins are very similar in structure. Both consist of a signal sequence followed by one variable and two constant immunoglobulin domains and are 58% similar in amino acid sequence. A critical difference is that the Lac protein is linked to cell membranes by a phosphatidylinositol anchor as opposed to the secreted Ama protein. Functions of Ama and Lac in neuronal development are still rather unclear. However, it is clear that Ama binds to Nrt and that this binding allows Nrt-expressing cells to aggregate.

To characterize potential Lac interactions with Ama and Nrt, a series of experiments was performed. The Lac gene was cloned from the *Drosophila* genome. Using *in vitro* mutagenesis, a new version of the Lac gene was created without the sequence coding for the membrane anchor to produce a secreted version of the Lac protein. To test the functional properties of Lac and secreted Lac (Lac sec) I utilized the S2 cell aggregation assay. *Drosophila* Schneider 2 (S2) cells normally exist as individual cells in suspension. However, if manipulated to express cell adhesion molecules, the S2 cells can form significant multicellular aggregates. Therefore, *Drosophila* S2 cells provide a perfect model with which to assay the ability of Lac to promote cell adhesion. Results confirmed previous findings that the secreted version of Ama binds to Nrt to form cellular aggregates. Also in agreement with past findings, Ama and Lac were able to act

individually as homophilic cell adhesion molecules. Both were able to induce homophilic aggregates, which served as a control for Ama and Lac protein production. S2 cells expressing the membrane-bound version of Lac and cells expressing Nrt were combined and assayed for cell aggregation activity. No mixed aggregates were observed which could indicate an inability of the Lac protein to bind to Nrt. The secreted version of Lac was also assayed for the ability to promote aggregation of Nrt-expressing cells. When Lac sec protein was added to media with Nrt-expressing S2 cells no significant multicellular aggregates were observed suggesting that the difference in amino acid sequence of the Ama protein compared to the Lac accounts for different Nrt-binding properties. To identify Ama sequences that confer the specificity of the Ama:Nrt interaction as well as Ama:Ama and Lac:Lac interactions, chimeric Ama-Lac genes were generated. In the future these chimeric proteins will be tested in the S2 cell assay for their different binding properties.

A new system for positive selection of transfected cells was tested and proven effective in the course of these experiments. This process utilizes resistance to the antibiotic Hygromycin B and is more advantageous than previous approaches used in the Seeger Lab due to the minimal cost of Hygromycin B.

INTRODUCTION

Nervous system development is dependent upon the correct wiring of neurons. In order to achieve proper connections, axons must often extend great distances to reach their final destination and synaptic targets. This process of axon guidance is both highly ordered and generally conserved in both vertebrates and invertebrates (1). Information from both vertebrate and invertebrate systems is critical to the study of axon guidance and will hopefully lead to a better understanding of the molecular and environmental cues and processes which make the axon's journey possible. In the future, the study of this process could possibly aid in such treatment as axon regeneration after trauma (1). During axon pathfinding a variety of extracellular and cell surface molecules may act as guidance cues. From the study of invertebrates such as Drosophila, many secreted ligands and cell adhesion molecules have been discovered that both promote and inhibit neurite outgrowth and aid in neuronal migration(1). This study will focus on three proteins that are expressed in the developing *Drosophila* embryonic nervous system; Amalagam, Lachesin, and Neurotactin (See Fig. 1).

Neurotactin (Nrt) is a transmembrane protein that is a member of the cholinesterase-homologous protein family. It is required for proper axon guidance in *Drosophila* neuronal development and is a heterophilic cell adhesion molecule (2). Schneider 2 cells expressing Nrt are able to interact with dissociated embryonic primary culture cells, and this ability first demonstrated its heterophilic adhesion properties (3). Nrt is a member of the serine esterase-like family of membrane proteins. This family of proteins is found in both vertebrates and invertebrates and members share a common domain structure. Nrt has a 500 amino acid extracellular that is related to the

cholinesterase family, followed by a 324 amino acid cytoplasmic domain. Nrt is expressed specifically during development, and is not observed in adult tissue. This suggests that its function is important for proper development (2). In fact, the loss-of-function Nrt mutant shows defects in axon guidance (4). Nrt is co-localized with Amalgam (Ama) throughout neuronal development indicating these proteins may interact during the process (2).

Ama is a secreted protein with 333 amino acids consisting of a signal sequence and three immunoglobulin-like domains (5). Ama is somewhat similar to many vertebrate neuronal cell adhesion molecules and is a member of the immunoglobulin superfamily. Ama first appears in a row of midline cells during gastrulation. Nrt is also expressed in these cells but also throughout the ectodermal layer by outlining the developing neuroblasts and their progeny (5). Ama is not found on the neuroblasts but Ama expression is identified on their progeny (5). By stage 13 of *Drosophila* development both Ama and Nrt are present in the fat body and throughout the central nervous system on cell bodies and axons. In later stages, both proteins accumulate in external sensory organ precursors in the peripheral nervous system (5). The accumulation patterns of Nrt and Ama throughout embryogenesis are very similar (2). Ama is a secreted protein and when added to media with Nrt-expressing S2 cells, the cells begin to aggregate (5). Therefore, Nrt interacts with Ama in vitro to promote cellular adhesion. It has been shown that only amino acids 347-482 of the Nrt extracellular domain is necessary for the Nrt-Ama interaction (2). It has also been shown that Ama can interact with itself in addition to the Nrt interaction. A membrane-bound version of Ama (Amalgam transmembrane, AmaTM) was created and tested in the S2

cell aggregation assay (See Fig. 1). Cells expressing AmaTM yielded homophilic aggregation (2). The Ama/Nrt cell adhesion process has been shown to be involved in axon-pathfinding (6). Specifically, previous results suggest that Ama/Nrt cell adhesion may be involved in signaling networks including Abelson tryrosine kinase in the neuronal growth cone (6).

Lachesin (Lac), another cell surface protein, may also play a role in *Drosophila* neurite outgrowth. Lac is a member of immunoglobulin superfamiy similar to Ama. Lac can function as a homophilic adhesion protein and mutations in Lac display defects in tracheal development, one of several tissues where Lac is expressed (7). Lac is also expressed on neuronal cells at the beginning of neurogenesis in the central and peripheral nervous system and on growing axons in the central and peripheral nervous system (8). This expression becomes restricted to a specific subset of neurons as development proceeds (8). Despite its known expression, the specific function of Lac in the nervous system is unknown (8).

Lac and Ama proteins are very similar in structure. Both consist of a signal sequence followed by one variable and two constant immunoglobulin domains and are 58% similar in amino acid sequence (8) (See Fig. 2). A critical difference is that the Lac protein is linked to cell membranes by a phosphatidylinositol anchor as opposed to the secreted Ama protein. Functions of Ama and Lac in neuronal development are still rather unclear. However, it is clear that Ama binds to Nrt and that this binding allows Nrt-expressing cells to aggregate. Due to the similarities of Ama and Lac and evidence for both to function as neuronal cell adhesion molecules, Ama and Lac could behave similarly throughout development. Specifically, we hypothesized that Lac could interact

with Nrt to promote cell adhesion similar to Ama. Therefore, the primary goal of these experiments is to determine if Lac and Nrt can interact to promote cell adhesion and further characterize the function of Lac in the developing nervous system. Another aim is to characterize the immunoglobulin domains of Lac and Ama and identify the regions necessary for their specific binding properties.

MATERIALS AND METHODS

Construction of secreted version of Lachesin protein (Lac sec)

In order to create a secreted version of Lac the Lac gene was isolated from the *Drosophila* genome. Specific primers were designed to amplify sequence coding for the three immunoglobulin domains. The sequence coding for the membrane anchor was not included in the sequence to be amplified, and therefore would be absent from the final protein product. The desired region was amplified by PCR with forward and reverse primers (lach-sec-for; sequence (5' to 3') AAA GAA TTC CGC GCT TGC AGG GTG TGG, lach-sec-rev; sequence (5' to 3') AAA GAA TCC TCA CAC GGG AAT GAT CGT CTC.) (PCR temperatures; 95°C, 58°C, 72°C) PCR product was cloned using TOPO cloning techniques and transformed into *E. coli* cells. Several individual colonies were chosen in order to grow separate cultures. DNA was isolated from cultures by performing boiling minipreps using Holmes-Quigly Buffer. The Lach-sec construct was isolated by digesting resultant DNA with specific enzymes. The identity of the amplified sequence was confirmed as Lach-sec with DNA sequencing. The construct was isolated in preparation for transfer to the pMET vector. The pMET vector and construct could then be expressed in S2 cells for the aggregation assay. The Lac sec insert was ligated with the pMET vector. The pMET vector + insert was transformed into *E. coli* cells, isolated, and clones with the correct orientation were identified using restriction digest mapping with digestion by EcoRV, EcoRI, NotI, StuI, EcoRV + SalI (double digest), and StuI +SalI (double digest).

Cell culture

Plasmid DNA encoding Hygromycin B (pHygro), UbGAL4 (Ub), UAS-GFP, and different experimental PMET constructs were isolated using Quiagen midiprep columns. Cell transfection experiments were performed. 75µl of water, 25µl of cellfectin (Invitrogen), and a vector solution containing a total of 10 µg of DNA was added to standard six well tissue culture plates containing S2 (Schneider 2) cells. Fetal Bovine Serum was added to 10% the following day. The following transfections into S2 cells were performed: pHygro (2 µg), Ub (4 µg), and UAS (4 µg); pHygro (2 µg) and PMET + Lac sec (8 µg); pHygro (2 µg) and PMET + Lac (8 µg); pHygro (2 µg) and PMET + Nrt (8 µg); pHygro (2 µg) and PMET + Ama (8 µg); pHygro (2 µg) and PMET + Amalgam Transmembrane (AmaTM) insert. The same transfections were performed substituting pHygro with a vector containing an α-amanitin-resistant RNA Polymerase II gene. 4 mL of old culture was passed into 20 mL of new media after 10 days to maintain cell lines.

Cell aggregation assays

To six well plates was added 3 mL each of respective cell cultures. $CuSO_4$ was added to Ama and Lac sec-expressing S2 cells and permitted to react overnight to induce production of proteins. $CuSO_4$ solution was added to S2 cell cultures transfected with pMET vector + insert to 0.7mM concentration. $CuSO_4$ induces expression from the metallothionine promoter in the pMET vector resulting in accumulation of the Lac sec and Ama proteins. In order to assay the effect of secreted proteins on cell adhesion, Lac sec and Ama producing cells were pelleted at low speed for 5 minutes in a centrifuge and protein-rich supernatant was removed. Appropriate cells to be assayed were also pelleted

in 3 mL aliquots and supernatant was removed. 3 mL of Lac sec or Ama protein solution was then mixed with the pelleted cells. Aggregation assays were conducted overnight on rotary shaker at 75 rpm. $CuSO_4$ was added to aggregation assays to induce production of membrane-bound proteins, AmaTM, Lac, and Nrt.

Heterophilic aggregation assays

In order to label a cell type, diI was added to the cell culture at a concentration of 5ul/5mL. The solution was permitted to sit overnight and then pelleted (in 3mL aliquots) to wash away unincorporated diI (5 minutes, low speed). Labeled cells were added to 3mL of a second cell type culture in a six well plate. These mixed-cell aggregation assays remained on a rotary shaker overnight at 75 rpm.

Construction of individual immunoglobulin domain DNA sequences of Ama and Lac for future chimeric proteins

In order to identify the critical domain on Ama for binding to Nrt S2 cells chimeric Lac-Ama proteins will be created for cell aggregation assay. In order to create the chimeric proteins, the DNA sequence of each domain must be isolated. Specific primers were designed to isolate and amplify the individual domains using PCR. The following primers were utilized (sequences 5'to 3'): lach3r; AAA GGA TCC GTG GGC CCA TTG GCT T, lach1r; AGG ACG GCG CAC CGA TAG CTT, lach2f; CCC GTC ATC TCG GAC AAC TCC, lach2r; TGC GAA CTC CAC CTC CAC GTT, lach3f; CCA GTG ATC ACC GTG CCG CGT, ama1r; CGG GGT CTT GAT CTG CAG GCT, ama-5prime-f; AAA GAA TTC GGC TAA CGC GAT CAA AAG ACT C, ama3-rev; GAT

GAC GGT CTG GAA GAG ATG, lach-GPI-f; CCC GTG TGC CCA CCG GCC TGT. The first Lac domain was isolated with lach-sec-f (primer used to create secreted version of Lac), forward, and lach1r, reverse. The frist two Lac domains together were isolated using lach-sec-f, forward, and lach2r, reverse. The second two Lac domains with the GPI anchor were isolated using lach2f, forward, and lach3r, reverse, while the isolation of the third domain with the GPI anchor required lach3f, forward, and lach3r, reverse. To create the sequence for the Lac GPI anchor alone lach-GPI-f, forward and lach3r, reverse were used. The DNA sequence for the first Ama domain was amplified with ama-5prime-f, forward, and ama1r, reverse. The first and second domains were isolated with ama-5prime-f, forward, and ama2r, reverse. Domains two and three were amplified with primers ama2f, forward, and ama3-rev, reverse. The third Ama domain was amplified with primers ama3f, forward, and ama3-rev, reverse. For PCR reactions VENT polymerase (New England Biolabs) was used due to higher proofreading activity than standard Taq polymerase. (PCR temperatures; 95°, five rounds at 50°/ twenty-five rounds at 62°, 72°) PCR products were purified and digested with restriction enzymes to prepare for ligation into the pMET vector. (DNA sequence for Lac domain 1 digested with EcoR1, sequence for Lac domains 1&2- EcoR1, sequence for Lac domains 2&3&GPI anchor- BamH1, sequence for Lac domain 3&GPI anchor- BamH1, sequence for Lac GPI anchor- BamH1, sequence for Ama domains 2&3- BamH1, sequence for Ama domain 3- BamH1, sequence for Ama domain 1- EcoR1, sequence for Ama domains 1&2- EcoR1.) All digested PCR fragments were then phosphorylated with kinase for ligations. Constructs will be ligated together to create complete chimeric proteins for future aggregation assay (see Fig. 14). Hybrid sequences in the pMET vector

will be transfected into S2 cells according to previous procedures and will similarly be subjected to the cell aggregation assay.

RESULTS AND DISCUSSION

In order to compare the functions of Lac and Ama a secreted version of Lac was necessary, because the lack of a membrane anchor on Ama is a significant difference between the two proteins. Furthermore, the secretion of the protein could allow for interactions not possible if attached to the membrane like wild-type Lac. In vitro mutagenesis was used to create a secreted version of Lac by deleting the final basepairs coding for the membrane anchor. PCR was used to amplify the desired sequence which was then cloned and ligated with the pMET vector. The sequence was purified and confirmed by DNA sequencing, which indicated no inappropriate mutations. Inside the pMET vector the Lac secreted (Lac sec) sequence could be expressed in S2 cells and induced with $CuSO_4$ to produce Lac sec proteins.

In order to characterize potential Lac interactions, a series of experiments was performed. *Drosophila* Schneider 2 (S2) cells normally exist as individual cells in suspension. However, if manipulated to express cell adhesion molecules such as AmaTM, the S2 cells can form significant multicellular aggregates (See Fig. 3). Therefore, *Drosophila* S2 cells provide a perfect model with which to assay the ability of Lac to promote cell adhesion. Nrt, Lac, Lac sec, Ama, and AmaTM sequences were all inserted individually into pMET vectors and these vectors were transfected into S2 cells. An individual cell line was created for each protein. A new system for positive selection of transfected cells, previously unused in the lab, was tested in these experiments as well. The use of a new antibiotic, Hygromycin B, for selection during transfection of S2 cells was proven reliable over the course of the experiments. Its use has become part of

standard procedure in the laboratory, and it is much less expensive than antibiotics used previously in the lab.

A control assay for protein production was performed. The pMET vector requires $CuSO_4$ to induce expression from the metallothionine promoter. S2 cells transfected with pMET-AmaTM and pMET-Lac showed no aggregation without $CuSO_4$ added. Cell lines formed large multicellular aggregates when $CuSO_4$ was added. Untransfected S2 cells do not aggregate with the addition of $CuSO_4$ (see below). The formation of aggregates is evidence for protein production. The aggregation observed is consistent with the prior evidence that both AmaTM and Lac act as homophilic cell adhesion molecules (5, 7) (See Fig. 4 & 5).

Previous findings demonstrate that secreted Ama can interact with membrane-bound Nrt to induce S2 cells expressing Nrt to aggregate (2). My results were consistent with previous studies. Nrt-expressing S2 cells were added to media containing Ama protein. For Ama media, Ama expressing cells were pelleted and supernatant with protein was removed and added to the Nrt cells. Nrt S2 cells alone were unable to aggregate, but the addition of Ama produced significant multicellular aggregates. The proposed model for Ama-Nrt interaction indicates Ama can bind to itself and bind simultaneously to a Nrt protein (2). Hence, an Ama protein pair connects two Nrt proteins instead of inducing a Nrt allosteric change to promote Nrt – Nrt interaction (see Fig. 6). Aggregation assays were conducted with S2 cells as a control. Un-transfected S2 cells and media containing Ama protein were combined and observed for aggregation. Also un-transfected S2 cells were assayed for aggregation alone. No aggregates were observed for either control aggregation assay (See Fig. 7).

Second, the question of whether Nrt can interact with Lac was addressed. Media containing the secreted version of Lac, Lac sec, and cells expressing Nrt were combined and assayed for cell aggregation activity. The presence of cell aggregates would be consistent with the ability of the Lac protein to bind to Nrt. However, no cell aggregates were observed. Also, no cell aggregates were observed in the S2 control or the S2 control with Lac sec media (See Fig. 8). This negative result suggests that Lac is unable to bind to Nrt in the same manner as Ama despite their similarities in structure. However, this negative result raises some issues. It is possible that Lac sec protein was not being produced as there was no positive assay to confirm production. Furthermore, a Lac antibody to label proteins was not available for these studies. Therefore, future experiments should include Western blot analysis with an antibody against Lac in order to confirm the production of Lac sec. The preliminary results do suggest that there is some difference in the Lac structure from Ama that hinders the binding of Lac to Nrt.

Heterophilic aggregation assays were used to assay the interactions of AmaTM and Lac cells, Nrt and Lac cells, and Nrt and AmaTM cells. In order to differentiate between cell types diI was added to a specific cell group and cells were permitted to absorb the lipophilic dye overnight (See Fig. 9). Photographs were taken using a brightfield microscope at 10X followed by a fluorescence microscope at 10X and 20X. The specific slide area observed remained constant throughout all three images for each assay. A control assay with a labeled and unlabeled group of AmaTM S2 cells confirmed the integrity of the mixed aggregation assay protocol and illustrated the appearance of mixed aggregates. Significant multicellular aggregates were observed in this control (See Fig. 10). Heterophilic assay was performed with Nrt and AmaTM cells. It was

hypothesized that these cells would form aggregates. Significant multicellular mixed aggregates were observed supporting the hypothesis that AmaTM can interact with Nrt. The model for cell adhesion involves the Nrt protein binding directly to AmaTM (see Fig. 11). Homophilic and heterophilic binding occurred simultaneously in this assay. AmaTM could bind to itself and to Nrt expressing cells. Interestingly, images showed large aggregates of mixed Nrt and Ama aggregates integrated with aggregates of AmaTM S2 cells. The AmaTM control for heterophilic adhesion showed a homogenous mixing of cells, whereas the Nrt and Ama aggregates showed some segregation (Compare Fig. 10c & 11c).

Nrt S2 cells and Lac S2 cells were assayed for heterophilic aggregation. In agreement with results of the original Lac sec assay, results showed Nrt S2 cells remaining individualized in solution, whereas Lac S2 cells were observed forming significant homogeneous aggregates. This confirmed the homophilic cell adhesion properties of Lac and supported evidence that Lac is unable to interact with Nrt and induce cellular aggregation (8) (See Fig. 12). Finally, to test for interaction between AmaTM and Lac cells, diI was added to AmaTM cells and both cell groups were mixed for aggregation assay. Results were very clear. Both Lac and AmaTM formed significant homophilic aggregates. However there was no evidence of mixed aggregates (See Fig. 13). Therefore, this suggests that Lac and AmaTM are unable to interact to promote cell adhesion. This also suggests that there is a significant difference in the binding domain of Lac versus Ama that is hindering Lac from behaving similar to Ama. Experimental results suggest that Lac is unable induce cellular aggregation with AmaTM or Nrt.

In order to characterize and locate the critical difference between Lac and Ama proteins a new series of experiments was proposed. Lac and Ama individually have three similar immunoglobulin domains. To locate the critical domain on Ama where the interaction with Nrt occurs to promote cell adhesion the domains will be separated and combined resulting in a series of Ama-Lac mixed proteins. In order to create the proteins, individual DNA sequences of sets of specific domains will need to be created by in vitro mutagenesis and amplified using PCR and cloning techniques. The resultant sequences in the pMET vector will be transfected into S2 cells. The S2 cells will be induced to produce proteins by activating the pMET promoter with $CuSO_4$. These chimeric proteins will be added to media with Nrt-expressing S2 cells and assayed for the ability to promote cellular aggregation. The DNA sequences for various domain combinations from each protein have been isolated by PCR. Genetic sequences for ten combinations of domains from Lac and Ama have been isolated (See Fig. 14). Five potential Ama-Lac hybrid clones may be created from these ten sequences, two secreted proteins and three with the Lac membrane anchor. For four control ligations both Lac and Ama protein domains will be recombined as a representation of their original wild-type forms. Currently, the next step is to ligate sequences of Lac and Ama together in the pMET vector. The chimeric sequence-pMET construct can then be transfected into S2 cells and induced to produce protein. However, the three-piece ligations required to combine different domains in pMET has proven difficult. Currently, we are perfecting this procedure and after successful ligations are achieved the products will be transfected into S2 cells.

In conclusion, control aggregation assay results reflect previous findings. Nrt binds to Ama and promotes cellular adhesion. Furthermore, results demonstrated that AmaTM can interact with Nrt-expressing cells to form heterophilic aggregates. Results suggest that Lac sec is unable to interact with Nrt to promote cell adhesion. However, the lack of positive control assay for Lac sec protein is an issue and future experiments should include Western Blot analysis with an antibody against Lac. In agreement with the negative findings, results showed that wild-type Lac-expressing cells do not interact with Nrt-expressing cells. AmaTM and Lac are also unable to interact and promote cell adhesion despite the similarity in their domain organization and amino acid sequence. In order to characterize the critical Ama domain for Nrt-binding properties and for the ability to induce cell aggregation, future aggregation assays have been purposed. The production of Lac-Ama chimeric proteins for S2 cell aggregation assay is an excellent model to characterize these critical Ama domains due to the similarities between Lac and Ama protein structures.

FIGURES

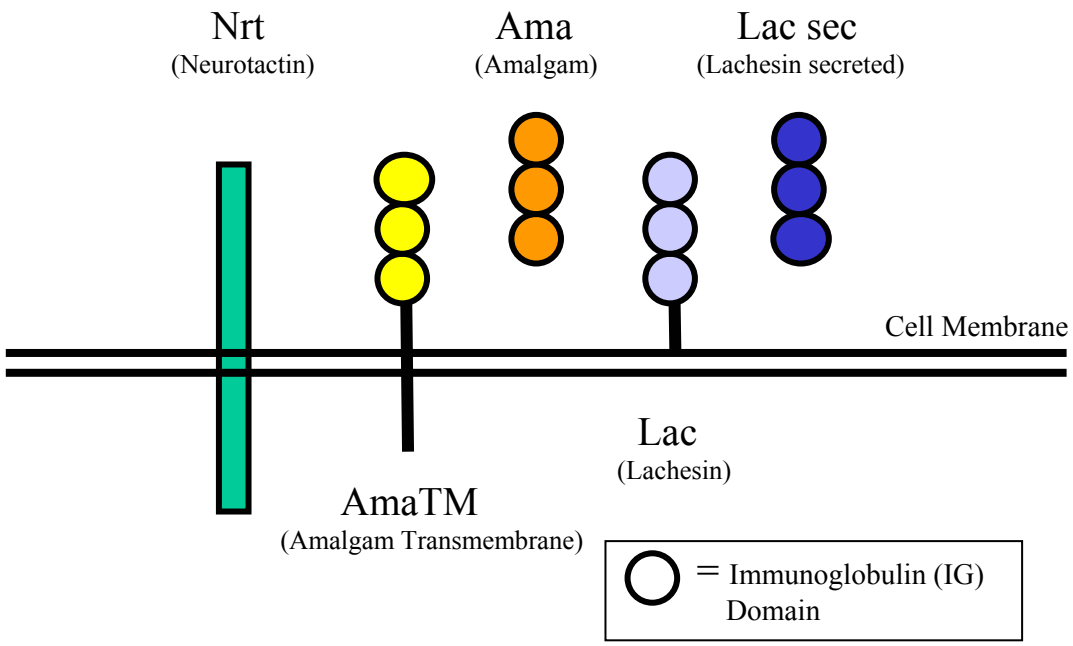


Fig.1 Key proteins: Previous research has shown that Ama binds to Nrt and promotes cell:cell adhesion during *Drosophila* neuronal development. Lac and Ama have extremely similar structures including three similar immunoglobulin domains. AmaTM and Lac sec proteins were created using in vitro mutagenesis.

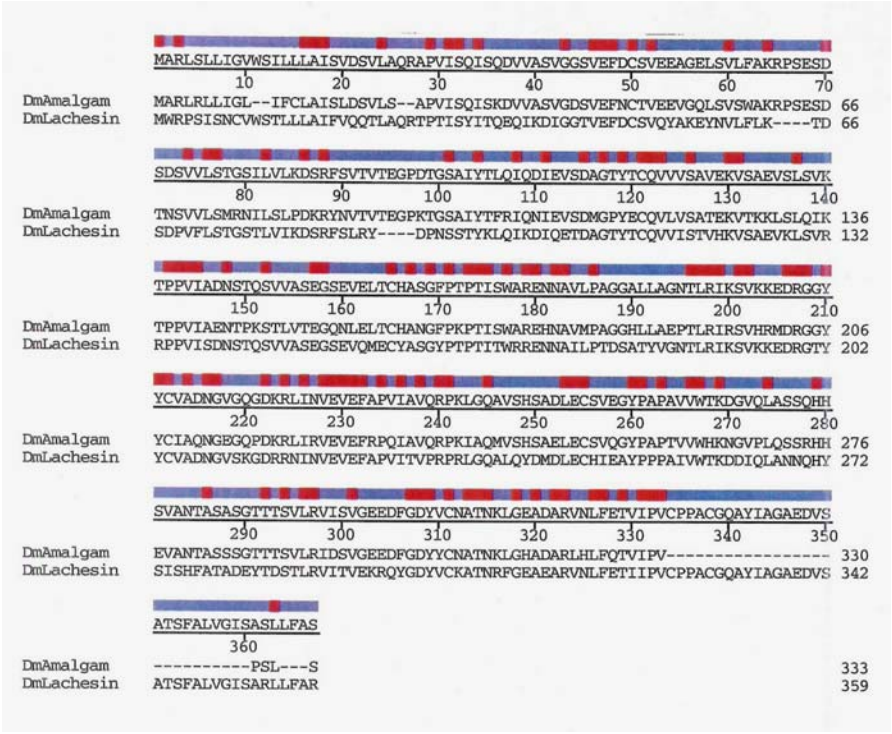


Fig. 2 Comparison of Lac and Ama amino acid sequences in *Drosophila melanogaster*: Lac and Ama proteins are 58% similar in amino acid sequence.

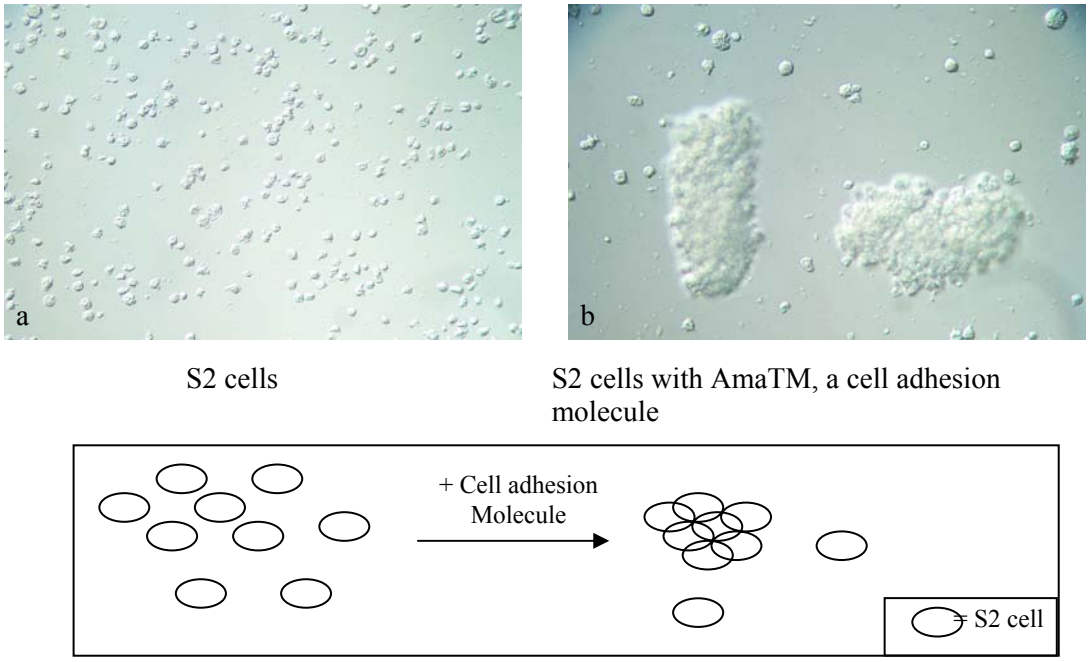


Fig. 3 The model: Schneider 2 (S2) cells: Drosophila S2 cells normally exist as individual cells in Suspension (a). However, if manipulated to express cell adhesion molecules, the S2 cells can form significant multicellular aggregates (b). Aggregation assays are conducted on a horizontal shaker overnight at 100 rpm.

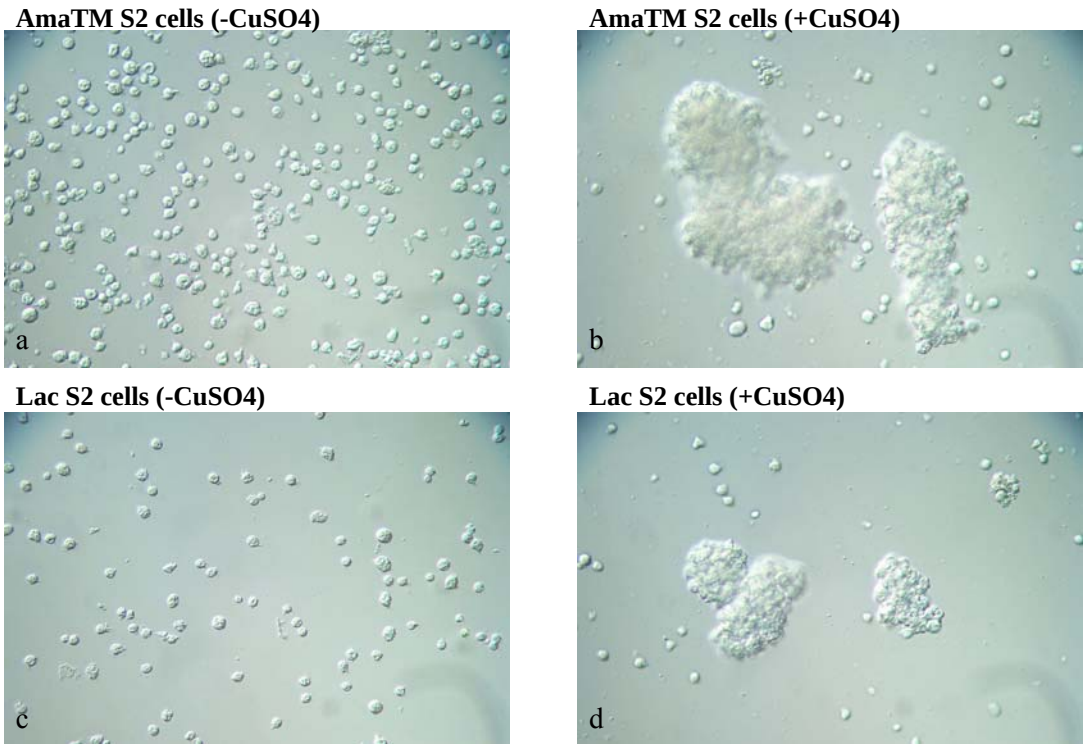
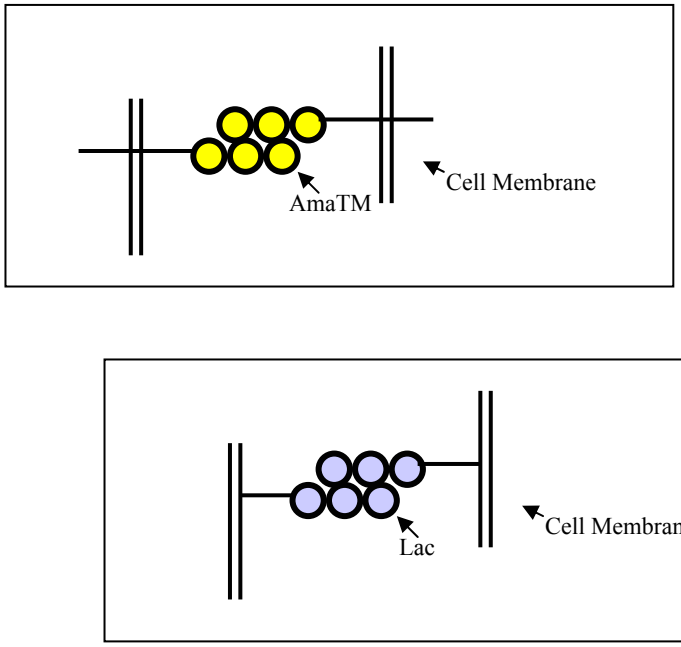


Fig. 4 Control for protein production: CuSO4 activates expression of the pMET vector containing the sequence for the desired construct. When CuSO4 is added, effects of protein products (in this case cellular aggregation) should be observed. Lac and AmaTM S2 cells showed no aggergation without CuSO4 (a,c). With CuSO4 significant aggregates were observed (b,d).



Possible models for cell adhesion

Fig. 5 Possible models for cell adhesion: Both AmaTM and Lac promote cell adhesion.

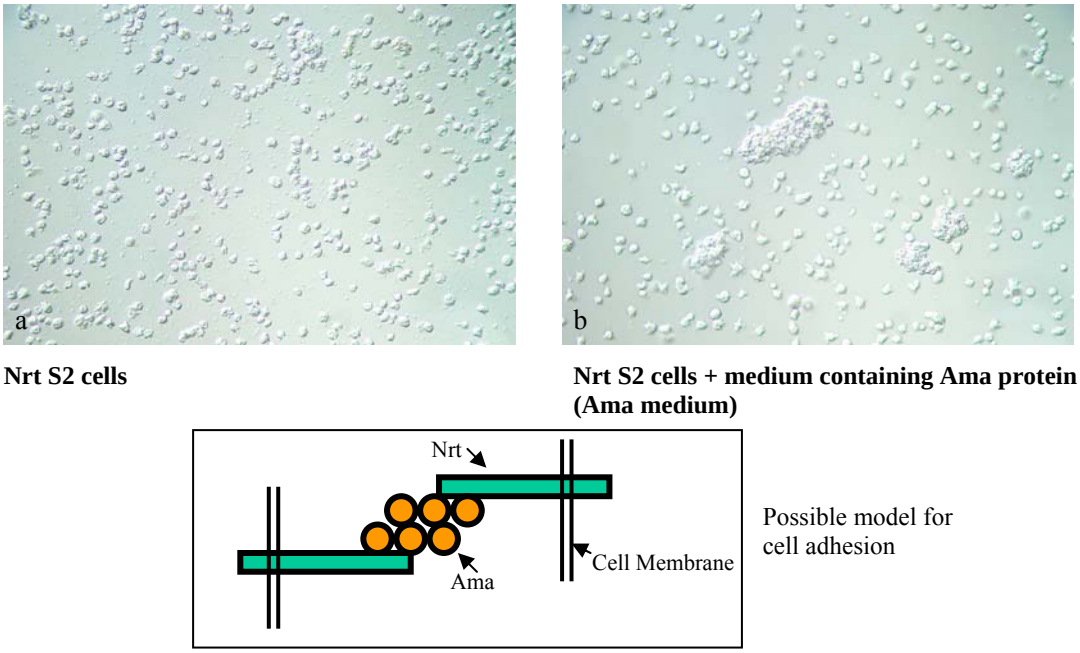
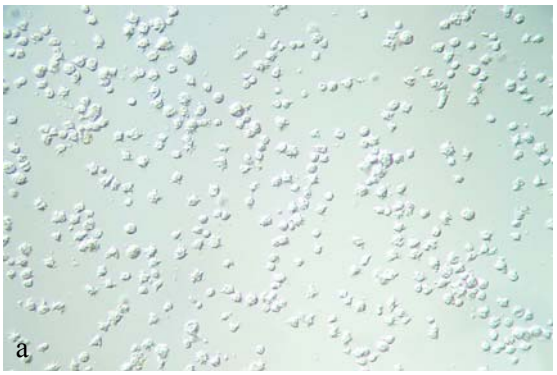


Fig. 6 Aggregation assay for Nrt cells and Nrt cells with Ama protein: Ama promoted cell adhesion in Nrt cells and caused cellular aggregation (b). However, individual Nrt cells were unable to aggregate without Ama (a). Ama and Nrt are known to physically interact by a number of other assays as well.

S2 cells + Ama medium



S2 cells

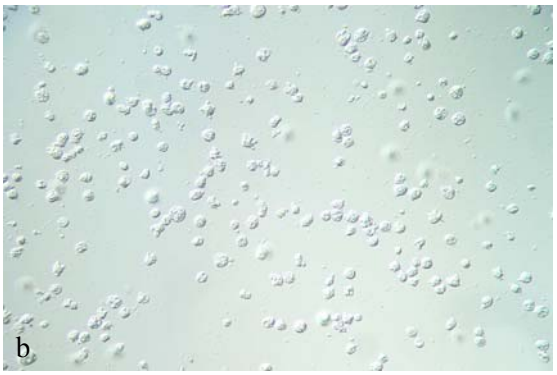
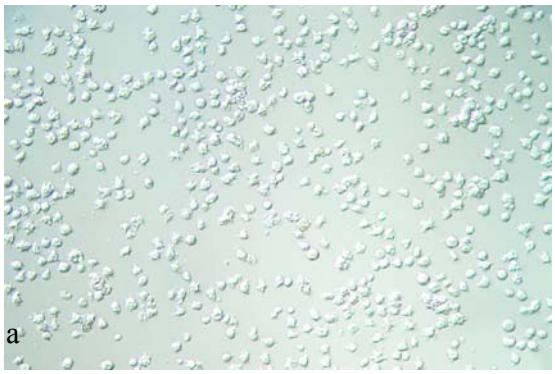
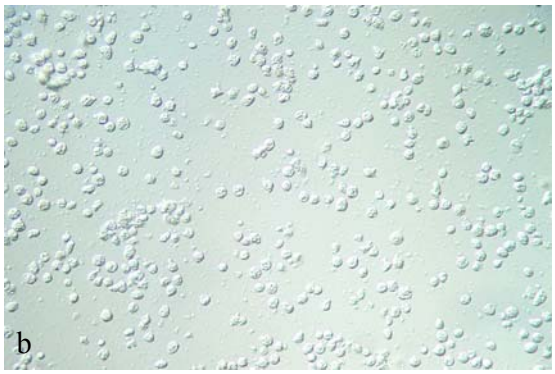


Fig. 7 S2 controls for cell aggregation assay: S2 controls were created for every cell aggregation assay. No aggregation was observed. For example, untransfected S2 cells + Ama-protein rich media showed no aggregation (a), and untransfected S2 cells alone showed no aggregation (b).

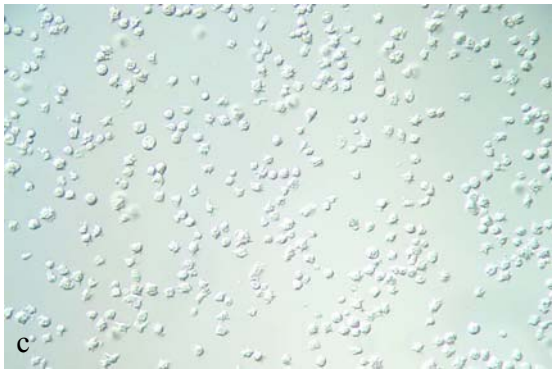


Nrt S2 cells + Lac sec medium



Nrt S2 cells

Fig. 8 Aggregation assay for Nrt cells with Lac sec protein: Preliminary results indicate that Lac sec does not interact with Nrt to promote cell adhesion. Even though Lac is similar to Ama they appear to have distinct activities. Nrt S2 cells did not aggregate in Lac sec medium (a). Controls of Nrt S2 cells without Lac sec media (b) and untransfected S2 cells + Lac sec media (c) showed now aggregation.



S2 cells + Lac sec medium

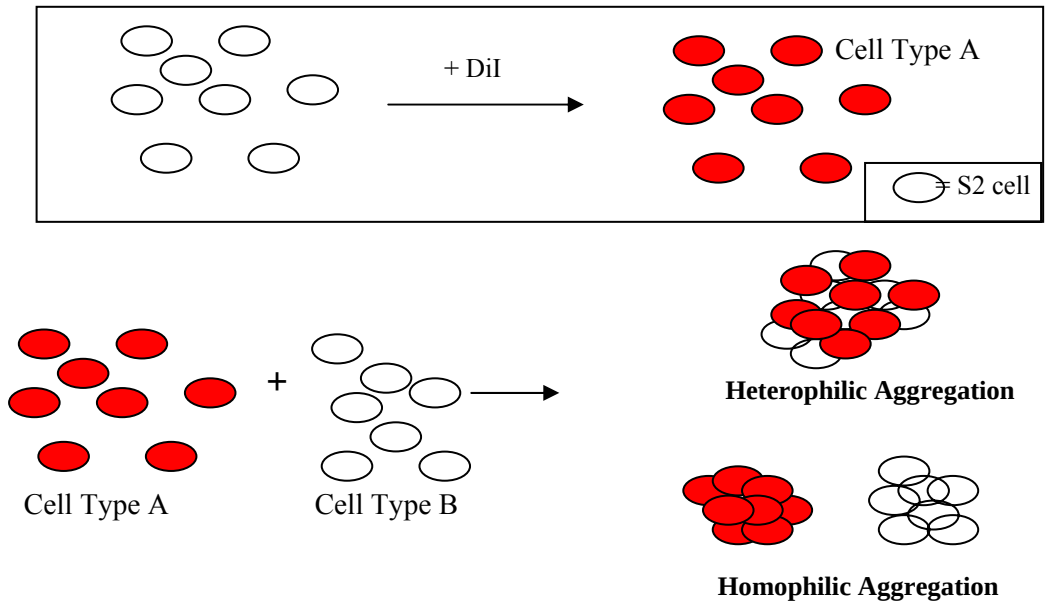


Fig. 9 Model for heterophilic aggregation assay: DiI was added to a specific cell type (concentration 5 uL / 5 mL) and left overnight. Labeled cells were then mixed with medium containing S2 cells of another cell type (unlabeled) and an aggregation assay was carried out as before.

AmaTM S2 cells +diI and AmaTM S2 cells (Brightfield 10x)

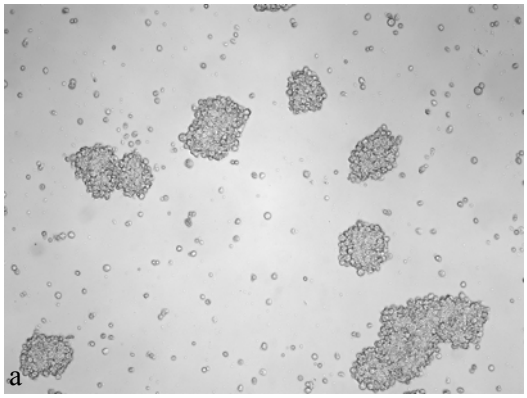
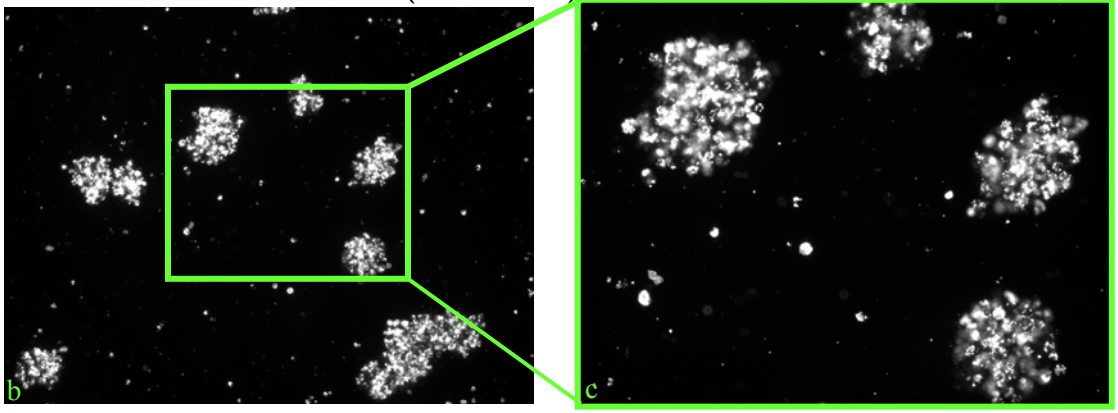


Fig. 10 Control for heterophilic aggregation assay: AmaTM S2 cells were labeled with diI and combined with an equal amount of unlabeled AmaTM S2 cells. These AmaTM aggregates represent true mixed aggregates of both labeled and unlabeled cells. 10x Brightfield (a) Fluorescent (b) 20x Fluorescent (c)

AmaTM S2 cells +diI and AmaTM S2 cells (Fluorescent 10x)

(Fluorescent 20x)



Nrt S2 cells +diI and AmaTM S2 cells (Fluorescent 10x)

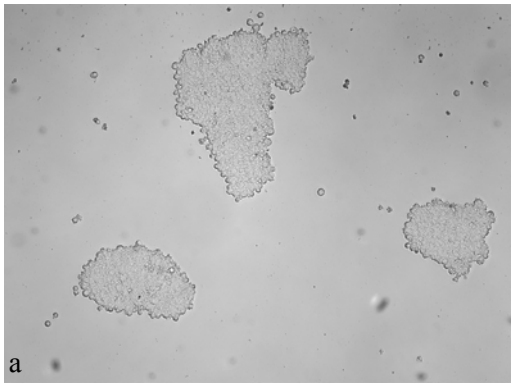
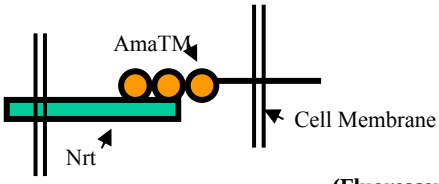
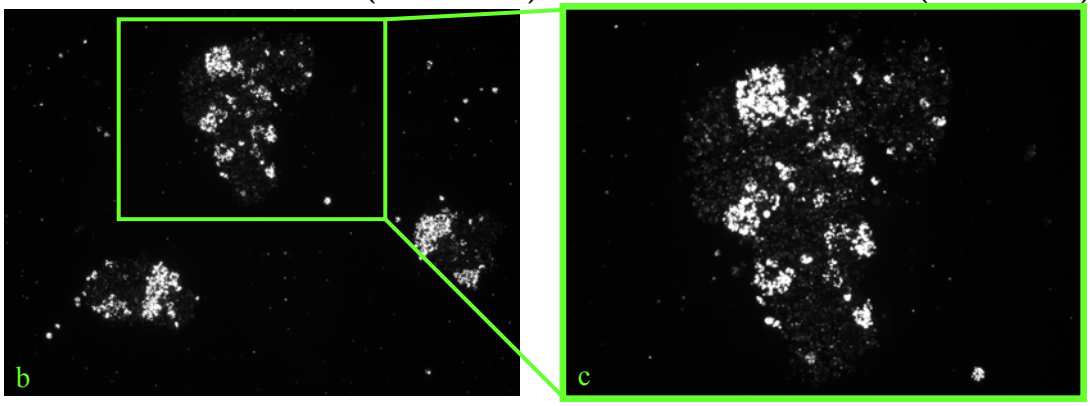


Fig 11 Heterophilic aggregation assay for Nrt and AmaTM: Nrt and AmaTM were able to form heterophilic multicellular aggregates. 10x Brightfield (a) Fluorescent (b) 20x Fluorescent (c) Possible adhesion model:



Nrt S2 cells +diI and AmaTM S2 cells (Fluorescent 10x) (Fluorescent 20x)



Nrt S2 cells +diI and Lac S2 cells (Brightfield 10x)

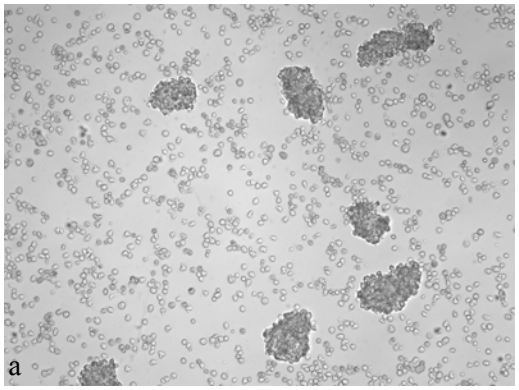
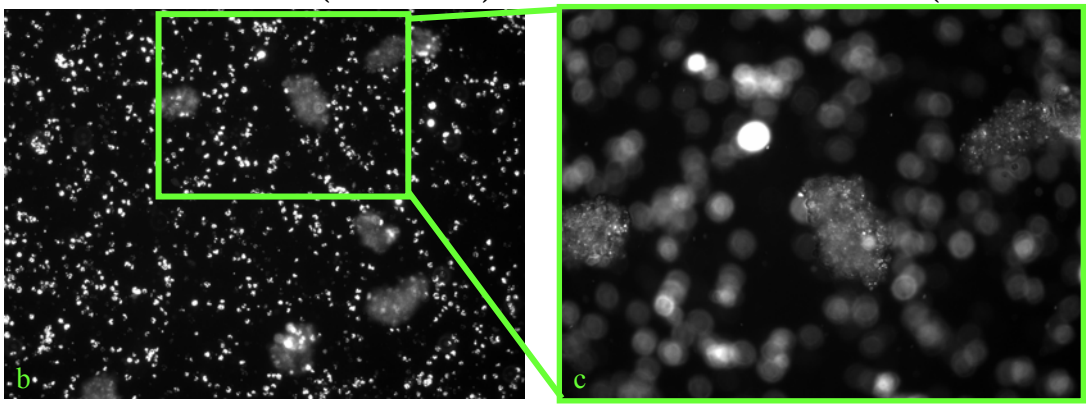


Fig. 12 Heterophilic aggregation assay for Nrt and Lac: Nrt and Lac were unable to form heterophilic multicellular aggregates. Lac formed homophilic aggregates. Nrt cells were unable to form aggregates consistent with previous results. Aggregates remain unlabeled with diI, while background cells (Nrt) fluoresce. These results are consistent with the previous finding that Lac sec was unable to facilitate multicellular aggregate formation with Nrt cells. 10x Brightfield (a) Fluorescent (b) 20x Fluorescent (c)

Nrt S2 cells +diI and Lac S2 cells (Fluorescent 10x)

(Fluorescent 20x)



AmaTM S2 cells +diI and Lac S2 cells (Fluorescent 10x)

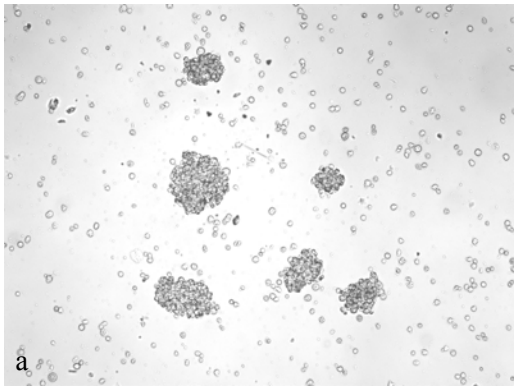
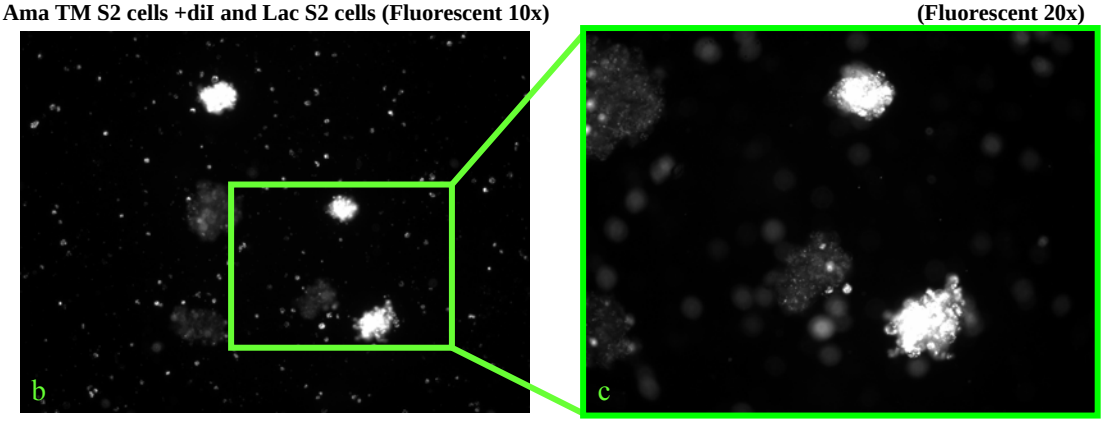


Fig. 13 Heterophilic aggregation assay for AmaTM and Lac: AmaTM and Lac were unable to form heterophilic multicellular aggregates. Both Lac and AmaTM formed homophilic aggregates. Labeled AmaTM cells form brightly fluorescing aggregates, while Lac aggregates are not visible with a fluorescent microscope. 10x Brightfield (a) Fluorescent (b) 20x Fluorescent (c)

Ama TM S2 cells +diI and Lac S2 cells (Fluorescent 10x) **(Fluorescent 20x)**



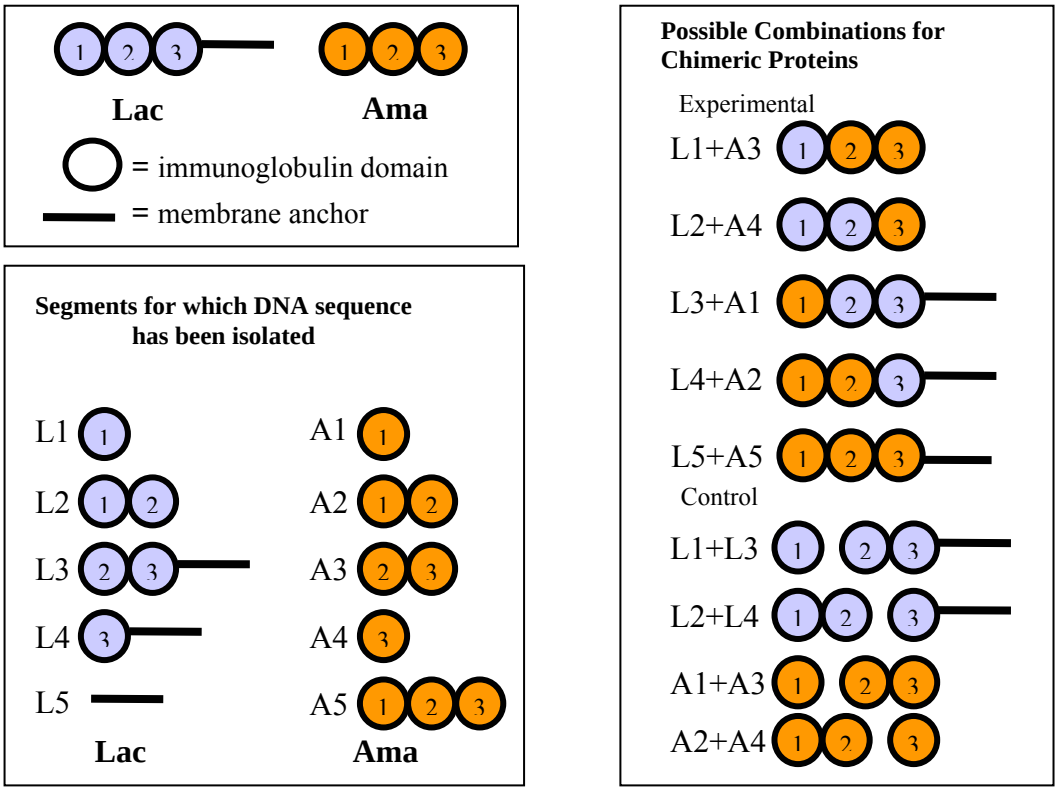


Fig. 14: Lac and Ama segments and potential hybrid proteins: DNA sequences of Lac and Ama segments have been isolated by PCR. Generation of hybrid clones in progress.

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